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## The Macrophage-Inducible C-Type Lectin, Mincle, Is an Essential Component of the Innate Immune Response to *Candida albicans*

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# The Macrophage-Inducible C-Type Lectin, Mincle, Is an Essential Component of the Innate Immune Response to *Candida albicans*<sup>1</sup>

Christine A. Wells,\* Judith A. Salvage-Jones,\* Xin Li,<sup>†</sup> Kelly Hitchens,\* Suzanne Butcher,\* Rachael Z. Murray,<sup>‡</sup> Anthony G. Beckhouse,\* Yu-Lan-Sandra Lo,\* Silvia Manzanero,\* Christian Cobbold,\* Kate Schroder,<sup>‡</sup> Bo Ma,<sup>§</sup> Sally Orr,<sup>§</sup> Lauren Stewart,<sup>§</sup> Daniel Lebus,<sup>§</sup> Peter Sobieszczuk,<sup>§</sup> David A. Hume,<sup>‡</sup> Jennifer Stow,<sup>‡</sup> Helen Blanchard,<sup>¶</sup> and Robert B. Ashman<sup>2†</sup>

The recognition of carbohydrate moieties by cells of the innate immune system is emerging as an essential element in antifungal immunity, but despite the number and diversity of lectins expressed by innate immune cells, few carbohydrate receptors have been characterized. Mincle, a C-type lectin, is expressed predominantly on macrophages, and is here shown to play a role in macrophage responses to the yeast *Candida albicans*. After exposure to the yeast in vitro, Mincle localized to the phagocytic cup, but it was not essential for phagocytosis. In the absence of Mincle, production of TNF- $\alpha$  by macrophages was reduced, both in vivo and in vitro. In addition, mice lacking Mincle showed a significantly increased susceptibility to systemic candidiasis. Thus, Mincle plays a novel and nonredundant role in the induction of inflammatory signaling in response to *C. albicans* infection. *The Journal of Immunology*, 2008, 180: 7404–7413.

The yeast *Candida albicans* is a widespread opportunistic pathogen; however, a high proportion of healthy individuals carry *C. albicans* as part of their normal gut and mucosal flora without any overt symptoms of infection. Disease is typically associated with a variety of factors that compromise innate or adaptive immune responses in the host. Invasive candidiasis represents a continuing threat in the hospital environment, with *C. albicans* alone accounting for approximately half of the mortality attributed to systemic forms of the disease (1).

In addition to external or iatrogenic factors that predispose to systemic or mucosal candidiasis, susceptibility is known to be associated with specific mutations or other experimentally induced genetic lesions that compromise the integrity of the host immune system (reviewed in Ref. 2). In this context, the pattern recognition receptors (PRRs)<sup>3</sup> of the innate immune system are of particular

relevance. The TLR family is the best characterized of the innate immune PRRs, with TLR2 playing an important role in responses against *C. albicans* (3–5). However, the mannose receptor (MRC1) (6, 7), Galectin-3 (8–10), and the dendritic cell-associated lectin (Dectin)-1 (11, 12) are also important in defense against fungal pathogens. Engagement of TLR2 by  $\beta$ -glucans on the surface of fungal pathogens leads to the activation of multiple signaling pathways in the host, particularly, the MyD88-dependent activation of the transcription factor NF- $\kappa$ B, and the P38 MAPK pathway, which act synergistically to promote the release of inflammatory cytokines such as TNF- $\alpha$ . Dectin-1 likewise recognizes  $\beta$ -glucans, and is a component of the phagocytic receptor complex (13). Dectin-1 contains an immunoreceptor tyrosine-based activation motif, a cytoplasmic domain that induces a second, TLR2-independent signaling cascade via spleen tyrosine kinase (14, 15). The role of Dectin-1 in vivo is still unresolved, as Dectin-1-null animals have been reported to be both susceptible (12) and resistant (11) to systemic *C. albicans* infection.

C-type lectins are a large and diverse class of carbohydrate-sensing receptors with an emerging role in innate immune surveillance. Mincle (also known as Clec4e and Clec5f9) is a novel 219aa type II transmembrane protein with a highly conserved C-type lectin domain (mouse and human Mincle share 85% protein similarity) (16). Mincle maps proximal to the NK cell gene complex on mouse chromosome 6 and human chromosome 12 in a cluster of four highly conserved group II lectins: Mincle, macrophage C-type lectin (Mcl), dendritic cell immunoreceptor, and Dectin-2 (17). Mincle was initially reported as a LPS-inducible protein (16), and was identified as a transcriptional target of the C/EBP $\beta$  (16), as

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<sup>3</sup> Abbreviations used in this paper: PRR, pattern recognition receptor; Dectin, dendritic cell-associated lectin; Mcl, macrophage C-type lectin; ES, embryonic stem;

WT, wild type; KO, knockout; Ct, cycle threshold; BMM, bone marrow-derived macrophage.

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well as IFN regulatory factor-8 (18), in mouse macrophages lacking the respective transcription factors. Although its function has not been previously described, it is known to be up-regulated in the lungs of mice infected with pneumococcal pneumonia or influenza A virus (19).

Macrophages are a major cell type involved in phagocytosis and clearance of both systemic and mucosal candidiasis, and we here demonstrate that Mincle, which is highly expressed on macrophages, is a receptor for *C. albicans*, and plays a significant role in the mammalian immune response against the yeast.

## Materials and Methods

### *C. albicans* cultures

*C. albicans* isolate 3630 (from a patient with cutaneous candidiasis) and isolate 3683 (from a patient with oral candidiasis) were obtained from the Australian Medical Mycology Reference Laboratory (Royal North Shore Hospital, Sydney, Australia). Isolate SC5314, originally derived from a patient with systemic candidiasis, was a gift from Dr. P. Sundstrom (Dartmouth Medical School, Hanover, NH). Yeasts were stored at  $-70^{\circ}\text{C}$  in 15% (v/v) glycerol in Sabouraud's broth and grown in Sabouraud's broth containing 4% peptone and 1% dextrose, with continuous agitation, for 24 h at  $28^{\circ}\text{C}$  before the assay. Growth at this temperature maintained the yeast in the blastoconidium phase (20).

### Mouse strains

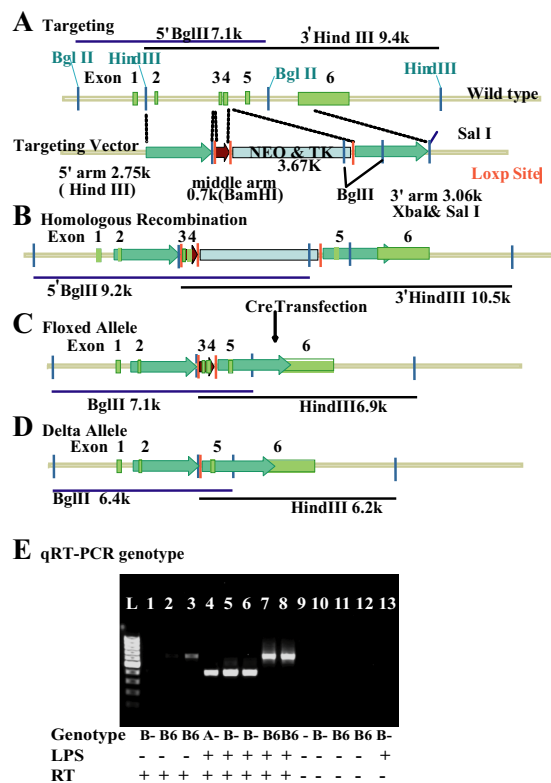
Specific pathogen-free BALB/c and C57BL/6J mice were obtained from the Animal Resource Centre (Perth, Western Australia), and housed under clean conditions at the Griffith University Animal Facility. Animal experiments were approved by the Animal Ethics Committee of Griffith University, and conducted in accordance with the National Health and Medical Research Council's Australian Code of Practice for Care and Use of Animals for Scientific Purposes (1997).

### Construction of recombinant knockout (KO) mouse lines *Clec4e<sup>MNA</sup>* and *Clec4e<sup>MNB</sup>*

Two independently floxed embryonic stem (ES) cell lines were used to generate germline deletions of the Mincle allele (*Clec4e<sup>MNA</sup>* and *Clec4e<sup>MNB</sup>*). The mouse C57BL/6J BAC clone RP23-284A5, which, based on the Ensembl database, spanned the Mincle gene, was obtained from BACPAC Resources Center at Children's Hospital Oakland Research Institute. Using the BAC DNA as a template, three PCR-amplified homologous fragments were sequentially cloned into the *plox* vector (Fig. 1), a conditional KO vector provided by Dr. J. Merth (Howard Hughes Medical Institute, University of California San Diego, San Diego, CA), containing three loxp sites (21). In the targeting vector (Fig. 1A), the 0.7-kb *Bam*HI middle fragment (containing exons 3 and 4) was flanked by the first and the second loxp sites; the PGK-Neo and HSV-Tk cassette was flanked by the second and the third loxp sites. The *Sal*I-digested linear targeting vector (Fig. 1B) was then transfected into the Bruce 4 ES cell, a C57BL/6 ES cell line from Orygene Pty. The homologous recombined ES clones carrying *Mincle<sup>F/itkneo</sup>* allele were identified by Southern blot. The selected ES cell clones were further transfected with the *pMC-Cre* vector encoding for the Cre recombinase and cultured with  $2\ \mu\text{M}$  ganciclovir. The resulting alleles are illustrated in Fig. 1, C and D. The ganciclovir-resistant ES cell sub-clones, bearing either the loxp-flanked exon 3 and 4 Mincle allele (*Mincle<sup>F</sup>* shown in Fig. 1C) or the deleted null allele (*Mincle<sup>A</sup>* shown in Fig. 1D), were selected and confirmed by PCR and Southern blot. ES cell clones with the *Mincle<sup>F</sup>* allele were archived in liquid nitrogen. ES cell clones with *Mincle<sup>A</sup>* allele were injected into BALB/cBy blastocysts to generate chimeric mice. Male chimeras were bred with C57BL/6 females. Resulting heterozygous  $F_1$  Mincle mice were crossed to produce homozygous mutants. Two resulting homozygous null mouse lines were generated: *Clec4e<sup>MNA</sup>* and *Clec4e<sup>MNB</sup>*. Genotyping of these lines was performed by PCR (on genomic and mRNA templates) and Southern blot, and phenotyping demonstrated that the two strains (*Clec4e<sup>MNA</sup>* and *Clec4e<sup>MNB</sup>*) were functionally identical.

### Primary bone marrow-derived macrophage (BMM) cultures

Bone marrow was derived from the femurs of male mice at 6–8 wk of age, and macrophages were differentiated under the selection of rM-CSF for 5 days as previously described (22). Macrophages were passaged on day 6 into experimental plates.



**FIGURE 1.** A schematic illustrating the construction of the targeting vector for the Mincle KO mouse. **A**, PCR-amplified 2.75-kb upstream fragment, 0.7-kb middle fragment, and 3.06-kb downstream homologous fragment to the Mincle BAC clone RP23-284A5 were cloned into the *plox* vector sequentially, with the middle fragment flanked by two loxp sites. **B**, Recombined allele after neomycin selection of transfected ES cells. Further transfection of ES cell clones with Cre recombinase resulted in a floxed (**C**) or deleted (**D**) allele. **E**, Expression of the WT allele in C57BL/6J or  $\delta$  allele in *Clec4e<sup>MNB</sup>* (MNB) mice. L, 100-bp ladder (fermentas), treatment of cDNA in lanes 1–8 is indicated by the chart below the 1.5% agarose/TAE gel. Genotype indicates the origin of the BMM, the WT strain (B6), and *Clec4e<sup>MNB</sup>* (MNB). LPS indicates presence (+) or absence (-) of 1-h 10 ng/ml LPS stimulation of the BMM. RT indicates the presence of reverse transcriptase in the cDNA reaction tubes. Lane 9, Water control. Lanes 10–13, Minus RT controls.

### Preparation of RNA

Bone marrow macrophages were harvested using a cell scraper and centrifuged at  $4^{\circ}\text{C}$  after coculture at  $37^{\circ}\text{C}$  with *C. albicans* yeast cells for 1 h in a small volume of culture medium to facilitate contact between yeasts and macrophages. Plates were placed on ice and washed three times with ice-cold PBS before extraction of total RNA using the RNeasy Midi kit (Qiagen) according to the manufacturer's instructions. RNA concentration and integrity was tested by lab-on-a-chip technology on the Bioanalyzer (Agilent).

### Microarray hybridization

Two aliquots of the same RNA sample were prepared for Cy3 and Cy5 labeling separately (Dye-swap experimental design). Cy3-dUTP and Cy5-dUTP (Amersham Biosciences) were directly incorporated into cDNAs from macrophage RNA samples, using Superscript III (Invitrogen). The labeled cDNAs from macrophages that were either untreated (0-h controls) or *Candida*-treated (1 or 6 h) were hybridized to 23K Compugen Mouse oligo chips produced by the Australian Research Council Special Research Center Microarray Facility (University of Queensland, Queensland, Australia; <http://microarray.imb.uq.edu.au>). Quality control on each print batch was assessed by Cy3 end-labeled random 10-mer hybridizations to check the DNA deposition and spot morphology and by hybridization with amplified RNA (universal mouse reference vs cell line NIH3T3) using the Aminoallyl MessageAmp kit (Ambion). In this experiment, each pair of



Table I. Primers used

Primer (bp)	Sequence	Product
Dectin-2 forward	CTGCCCAATCACTGGAAGT	193
Dectin-2 reverse	ATCCGAAAGACCCAGGAAGT	
MCL forward	TTGTGACCCAGCTTTTGGAT	
MCL reverse	TCCAGACCCATTTTTCATGG	195
Mincle forward	TGCTACAGTGAGGCATCAGG	
Mincle reverse	GGTTTTGTGCGAAAAGGAA	
DCAR-1 forward	TGCTAGACACTGCTGCTGGT	150
DCAR-1 reverse	TCCAGCCCCACATAGTTTTC	
DCAR-2 forward	GTTGTGGGATACAGGCCATC	
DCAR-2 reverse	GATATCATTCAGCCCCATC	157
HPRT forward	GCTGGTGAAGGACCTCT	
HPRT reverse	CACAGGACTAGAACACCTGC	

samples were hybridized twice with dye swap, and three independent biological replicate samples were used to generate six hybridization replicates for analysis.

### Image and data analysis

Microarrays were washed and spun dry, and scanned immediately at a resolution of 5  $\mu\text{m}/\text{pixel}$ . Raw TIFF files were imported by pair (Cy5 and Cy3 channel) to DigitalGENOME molecularware software. Intensities of every spot and its local background were calculated and low confidence spots were flagged based on morphology and coefficient of variation. The resulting annotation files were then exported to GeneSpring 7.2 for further data analysis. Background correction, dye swap, and LOWESS normalization were performed to generate a ratio of *Candida*-treated/untreated cells for each gene. Then, genes were filtered on image flags and presence across the series of replicas to remove unreliable data. Differentially expressed genes were identified using *t* tests with a *p* value cutoff of 0.05, with Benjamini and Hochberg false discovery rate applied. Annotation and ontology analyses were conducted using the National Institutes of Health National Institute of Allergy and Infectious Diseases online tool of Database for Annotation, Visualization, and Integrated Discovery (<http://david.niaid.nih.gov/david/version2/index.htm>).

### Validation of gene expression by quantitative real-time PCR

RNA were isolated from BMMs from 6-wk-old male Clec4e<sup>MNB</sup> or C57BL/6J (wild-type (WT)) mice using Qiagen RNeasy minispin columns. cDNA was prepared using oligo dT and Superscript III (Invitrogen) according to the manufacturer's directions. Quantitative real-time PCR mixes were prepared using the SensiMix (Quantace) and EvaGreen dye (Quantace) quantitative RT-PCR reagents. Primer sequences are shown in Table I.

Cycling conditions were 95°C 15 s, 60°C 20 s, 72°C 15 s in a Rotorgene 6000 (Corbett). The expression ratio was determined according to the Pfaffl method (23):  $\text{ratio} = E(\text{target})^{\Delta C_t} / E(\text{reference})^{\Delta C_t}$ , where  $C_t$  is the cycle threshold.

### Ab design

An affinity-purified polyclonal Ab against an extracellular domain of Mincle, conserved between mouse and humans, was raised in rabbits by a commercial supplier (Bio Synthesis) using the hydrophilic peptide TQE EQEFLFRTPKPKRKEF. The Ab was supplied at a stock concentration of 0.6 mg/ml<sup>-1</sup>.

### Immunofluorescence staining

RAW264.7 mouse macrophages were cultured as previously described (24). Macrophages plated at a low density were grown for 2 days on coverslips and incubated with *C. albicans* at a ratio of 10:1 (yeast:macrophage) for 2–40 min and fixed for 5 min in ice-cold methanol. Immunofluorescence staining was performed as described previously (24). Briefly, cells were blocked with PBS containing BSA, then incubated for 1 h with primary Ab followed by the appropriate secondary Ab conjugated to Alexa-488 or Cy3 (Amersham biosciences). CD11b Abs were purchased from Abcam. Coverslips were mounted on glass slides in 50% glycerol/1% *N*-propylgallate in PBS, then examined using either an Olympus AX70 microscope or a Zeiss LSM510 META confocal microscope (Carl Zeiss Microscope Systems) equipped with a  $\times 100$  oil objective.

### ELISA for TNF- $\alpha$

Primary BMM or RAW264.7 cells were grown in a 96-well plate. Anti-Mincle Ab was serially diluted (from a concentrated stock at 0.6 mg/ml) in RPMI 1640 without serum. The cells were preincubated with the appropriate dilution of Ab for 15 min at 37°C in 5% CO<sub>2</sub> before the addition of *C. albicans*. Eight replicates were performed for each dilution (one column of a plate). Supernatant was collected from cells after 2-h incubation at 37°C in 5% CO<sub>2</sub>, and TNF- $\alpha$  concentration measured using the mono-mono TNF ELISA kit (BD Bioscience) according to the manufacturer's instructions.

### Phagocytosis assay

The phagocytosis protocol was based on that previously described by Dhuley (25). Primary BMM or RAW264.7 cells were grown on sterile coverslips in a 24-well plate. Where appropriate, Mincle Ab was added to RPMI 1640 in serial dilutions from the concentrated stock (0.6 mg/ml). Media was removed from the cells and 50  $\mu\text{l}$  of each Ab dilution was added to the respective wells in quadruplicate. Following preincubation with Mincle Ab, 200  $\mu\text{l}$  of yeast suspension was added to each well, at an infectivity ratio of five yeasts per macrophage, and incubated at 37°C, 5%CO<sub>2</sub> for 1 h. To remove any nonadherent yeast, the cells were washed in RPMI 1640 and incubated for a further 30 min at 37°C. Following infection, the cells were washed in cold PBS, fixed with 1% paraformaldehyde, and stained with hematoxylin for 15 min followed by staining with eosin. The cellular material was examined using bright field microscopy, counting at least 200 macrophages in different fields across each coverslip. Each assay was replicated three times with a minimum of four discs for each dilution. The percentage of macrophages phagocytosing at least one yeast cell (*P*) was determined along with the average number of yeast cells observed within the macrophages (*F*). The phagocytic index (*I*) was calculated by  $I = P \times F$ , representing the mean number of *C. albicans* ingested per phagocytosing macrophage (26).

### Expression of full-length recombinant human Mincle protein in HeLa cells or RAW264.7 cells

Full-length human Mincle protein was cloned into the N-terminal xpress-epitope tag vector pEF1/HISB (Invitrogen), using primers that flanked the open reading frame. Primer sequences were: Mincle NotR: GCATGCGG CCGCTTAAAGAGATTTTCCTTTGTTCAAAGG; Mincle KpnF: GCAT GGTACCCACCATGAATTCATCTAAATCATCT, with the restriction enzyme sites Kpn and *Not*I, respectively, indicated in italics. Purified endotoxin-free plasmid DNA was prepared using the UltraClean mini plasmid kit (MoBio Laboratories; Geneworks). HeLa cells were grown as monolayer cultures in DMEM (Invitrogen) containing 10% (v/v) FBS (Serum supreme; BioWhittaker), 2 mM L-glutamine, 100  $\mu\text{g}/\text{ml}$  penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin and maintained at 37°C with 5% CO<sub>2</sub>. Cells were seeded on to sterile coverslips in 24-well culture plates at a concentration of  $1.25 \times 10^5$  cells/ml in complete culture medium, and grown for 18–24 h. For transient expression of Mincle, HeLa cells were transfected with 0.5  $\mu\text{g}$  of plasmid/well using Lipofectamine Plus (Invitrogen) according to the manufacturer's standard procedure. After 20-h treatment, cells were washed and subsequently cultured for 24 h in DMEM containing 2% FBS before treatment as indicated in the figure legends. RAW264.7 cells were cultured in RPMI 1640 (Invitrogen) containing 10% (v/v) Serum Supreme (Lonza), 2 mM L-glutamine plus 100  $\mu\text{g}/\text{ml}$  penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin, and maintained at 37°C with 5% CO<sub>2</sub>. Cells were transfected using the Amaxa Biosystems Nucleofector kit V according to the manufacturer's instructions. Transfected cells were plated at  $5 \times 10^5$  cells/well into a 24-well plate containing sterile coverslips, and cultured for 24 h (as above) before fixation before treatment as described in the figure legends. Cells were then fixed for 30 min in 4% paraformaldehyde in PBS, and washed three times in PBS containing 0.1% (v/v) Triton X-100. Cells were blocked with Tris-HCl buffer (100 mM, pH 7.8) containing 0.5% fish gelatin and 50% normal goat serum (27). Rabbit anti-Mincle or mouse anti-xpress-HRP Ab, diluted in blocking buffer, was added to cells for 1 h, washed, and secondary Abs (Alexa Fluor goat anti-mouse 546 and Alexa Fluor goat anti-rabbit 488 obtained from Molecular Probes) were added. Nuclei were stained with Hoechst 33342 (10  $\mu\text{g}/\text{ml}$  working stock; Invitrogen). Cell preparations were viewed using a AxioCamMR and Apotome-Cam cameras mounted on a Zeiss Axio Imager Z1 microscope using  $\times 63$  oil objectives. To avoid bleed-through from different channels, sequential capture of each image was performed on double-labeled samples.

### Bacterial expression and purification of recombinant mouse Mincle protein

The extracellular domain of Mincle protein (amino acids 46–214) was cloned into the pET14b vector using forward (5'-ATG ACT Aca'tat gAC ATA TCG CAG CTC TCA AAT T-3') and reverse primers (5'-TGA g'ga tcc TTA GTC CAG AGG ACT TAT TTC-3') which contained restriction sites (italicized) for *Bam*HI and *Nde*I, respectively, for directional cloning. Bacterial expression proceeded at 25°C in Luria-Bertani broth (supplemented with chloramphenicol (34 µg/ml) and ampicillin (100 µg/ml)), with IPTG induction. Mincle protein expression and purification is described in detail elsewhere (A. Bugarcic, submitted for publication). Briefly, Mincle protein was found to be insoluble, so it was refolded as previously described for the C-type lectin Dectin-1, using a dilution protocol (15, 28). Denatured and purified protein was diluted 200-fold into the refolding buffer (200 mM Tris pH 8, 10 mM EDTA, 1M L-arginine, 1 mM GSH, 0.1 mM GSSG, 0.1 mM PMSF), then concentrated by centrifugation at 4°C at 4000 rpm in a 5-kDa cutoff Amicon centrifugation device (Millipore). Correct folding of the recombinant protein was determined by recognition of the tag and CTLD epitopes in their native state by Ab (ELISA analysis) and dynamic light scattering to determine aggregation state (A. Bugarcic, submitted for publication). The binding of recombinant Mincle protein to yeast was determined by an ELISA method. Briefly, soluble yeast extract was prepared as previously described (29) and the protein content was determined by a Bradford protein estimation. A Spectra-Plate-96 HB (PerkinElmer) was coated with 250 µl of the desired concentration of soluble yeast extract from heat-killed *C. albicans* or *Saccharomyces cerevisiae* strain BY4714. All samples were diluted in 50 mM carbonate buffer. Samples (100 µl) were incubated in duplicate at room temperature for 3 h, and washed three times in ELISA wash buffer (PBS, 0.01% (v/v) Tween 20 (Sigma-Aldrich)). Wells were blocked with 300 µl of 1% BSA in 1× PBS with 0.01% Tween 20 for 2–3 h. The recombinant protein was added at 10 µg/ml, and detected with an anti-HIS Ab (Cell Signaling Technologies) diluted according to the manufacturer's specifications to 1/2000 in ELISA wash buffer, and incubated overnight at 4°C. Secondary Ab (100 µl goat anti-rabbit; BD Pharmingen) was added at a 1/4000 dilution of 1 mg/ml stock in ELISA wash buffer, and incubated for 2–3 h at room temperature. Following the addition of 200 µl of FAST OPD Detection substrate (Sigma-Aldrich), the plates were incubated in the dark for 30 min. The reaction was stopped by the addition of 50 µl/well of H<sub>2</sub>SO<sub>4</sub>, and the absorbance of the plate read at 492 nm for 0.1 s (Vickor 3 microtiter plate reader; PerkinElmer).

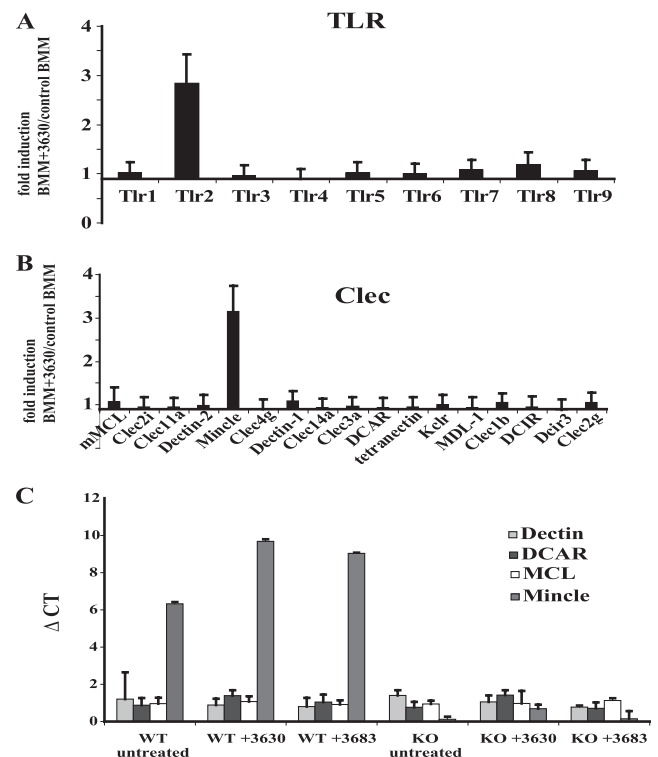
## Results

### Mincle and TLR2 are coordinately induced in mouse macrophages in response to *C. albicans*

Mincle was initially implicated in host responses to *C. albicans* infection in a microarray screen of primary mouse macrophages exposed to live *C. albicans* strain 3630 for 1 hour. The expression of host PRRs, including the TLR and C-type lectin families, was assessed before and after yeast exposure (Fig. 2), and a shared pattern of expression was observed between Mincle and TLR2. The induction of Mincle mRNA in primary macrophages stimulated with *C. albicans* was confirmed by quantitative real-time PCR. No other Dectin family members, including Dectin-1 or Dectin-2, were expressed at detectable levels in the BMMs. It was concluded that coregulation of Mincle and TLR2 implied a functional role for Mincle in the response of macrophages to *Candida* infection.

### Mincle is expressed on the surface of macrophages and is recruited to the phagosome

To assess the role of Mincle protein in the macrophage response to *C. albicans*, an affinity-purified polyclonal Ab was raised against the predicted ligand-binding domain of Mincle, in a region of the C-type lectin cup that was highly conserved between mice and humans. The Ab did not specifically recognize denatured recombinant or WT protein by Western blotting, therefore its specificity for native protein was confirmed by immunofluorescence. Recombinant mouse or human protein was overexpressed in HeLa cells, a mammalian cell that does not normally express Mincle on the cell surface.



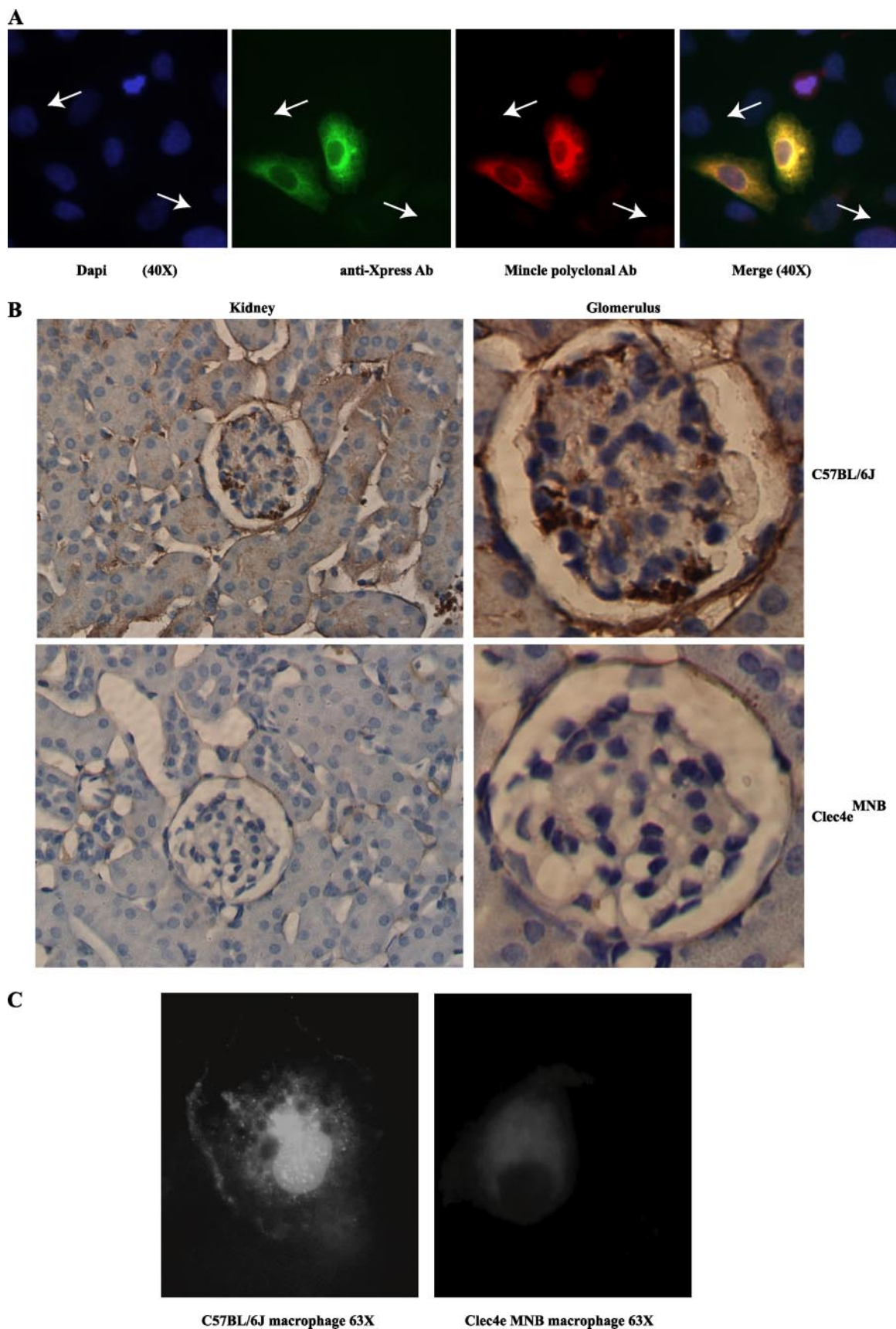
**FIGURE 2.** Gene-profiling data demonstrating coordinate expression of TLR2 and Mincle in mouse BMM, after exposure to live *C. albicans* for 1 h. **A**, Expression data for members of the TLR family, plotted as a normalized ratio of stimulated/unstimulated macrophages. The data are presented as mean ± SE of eight biological replicates. **B**, Expression of the myeloid C-Type lectin family plotted as a normalized ratio of stimulated/unstimulated macrophages. The data are presented as mean ± SE of eight biological replicates. **C**, Expression of a subset of the myeloid C-Type lectin family in mouse BMMs exposed for 1 h to *C. albicans* isolates 3630 and 3683, as assessed by quantitative real-time PCR. WT indicates C57BL/6/J BMM; KO indicates Clec4e<sup>-/-</sup> BMM. From left to right in each series: Dectin-1 (light gray); dendritic cell immunoactivating receptor (black); Mcl (white), and Mincle (dark gray). ΔCt is normalized to the housekeeping gene *HPRT*. The data are presented as mean ± SE of three biological replicates.

Fig. 3A demonstrates that the Ab bound only to HeLa cells expressing recombinant Mincle protein, as the anti-Mincle Ab was detected only in the cells also expressing the anti-xpress epitope, so demonstrating that the Mincle Ab detected the native protein in a specific manner. Likewise, the Mincle Ab did not bind to either tissues (Fig. 3B) or isolated BMM from Mincle gene KO mice (Fig. 3C).

The Mincle Ab was next used to establish patterns of localization of the native Mincle protein during phagocytosis of yeast by RAW264.7 cells. Immunofluorescence imaging showed that endogenous Mincle was located primarily on the cell surface (Fig. 4A), and during yeast ingestion Mincle was found concentrated at the nascent phagocytic cup (Fig. 4B). This pattern of expression was also seen in primary BMMs, confirming colocalization of Mincle to phagocytic cups forming around yeast particles (Fig. 4C). The distribution of Mincle protein in the mouse macrophage-like cell line RAW24.7, and in WT macrophages, was typical of an integral membrane protein, in a pattern similar to other macrophage cell surface proteins, including CD11b (Fig. 4A).

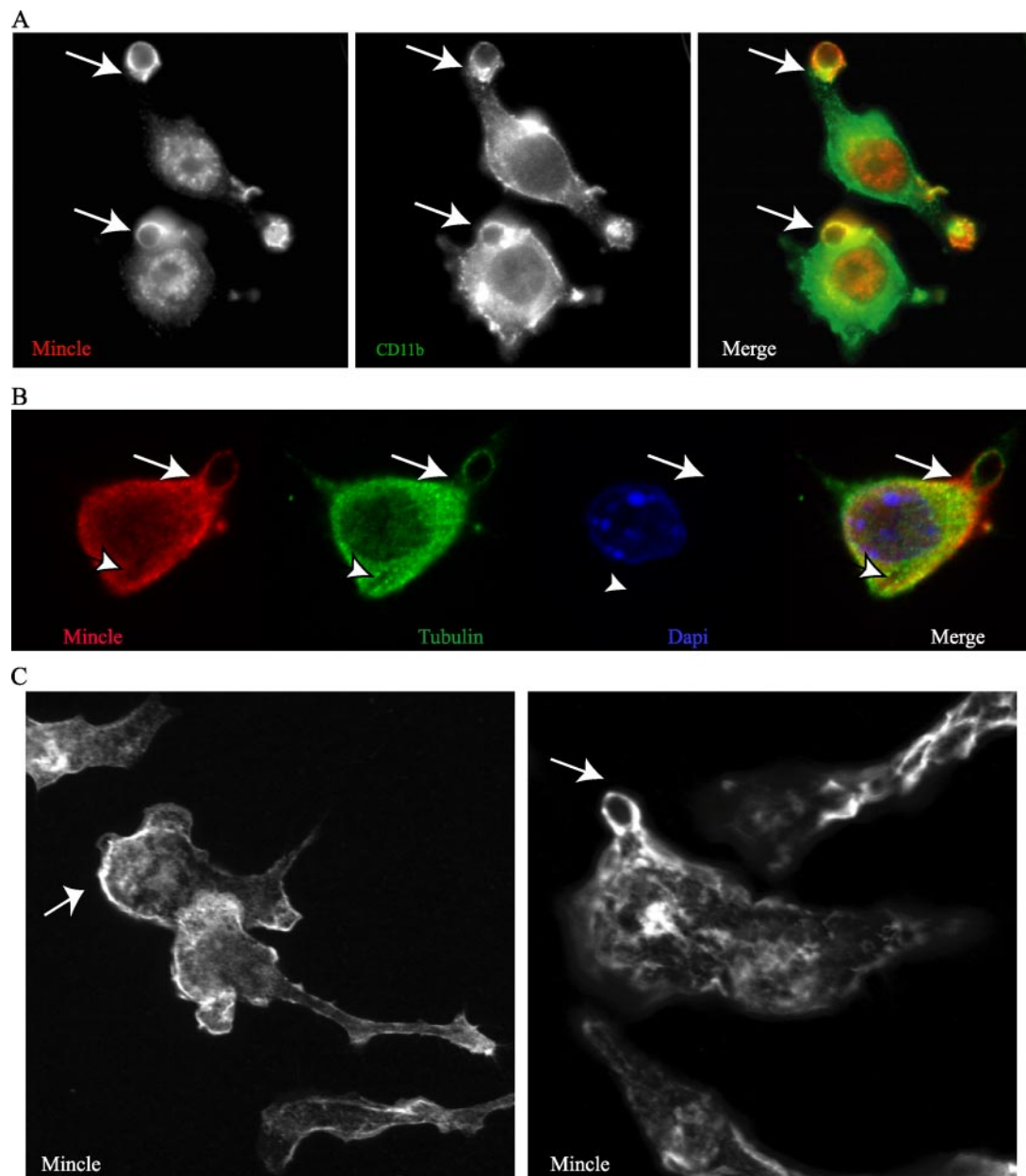
### Mincle binds *C. albicans* and induces TNF-α production in macrophages

The pattern of expression of Mincle protein suggested a functional interaction with *C. albicans*, so the extracellular (C-type lectin)



**FIGURE 3.** Specificity of the Mincle polyclonal Ab, as demonstrated by immunofluorescence. *A*, Recombinant Mincle protein expressed in HeLa cells (white arrows indicate untransfected cells) ( $\times 40$  original magnification). *B*, Native Mincle protein expressed in kidney from WT C57BL/6J mice (*upper panel*), but not MNB (Clec4e<sup>-/-</sup>) mice (*lower panel*) ( $\times 20$  original magnification). *C*, Native Mincle protein expressed in BMM from WT C57BL/6J mice (*left panel*), but not MNB (Clec4e<sup>-/-</sup>) mice (*right panel*) ( $\times 63$  original magnification).





**FIGURE 4.** Mincle is a cell surface protein that localizes to the nascent phagocytic cup. *A*, Expression of Mincle in RAW264.7 macrophages exposed to *C. albicans* stained with the Mincle polyclonal Ab at a 1/100 dilution (red), or the macrophage cell surface marker CD11b (green) ( $\times 100$  original magnification). *B*, Recombinant human Mincle (red) overexpressed in RAW264.7 cells, accumulated at the phagocytic cup (white arrows) after exposure to *C. albicans*. Tubulin is stained green and nuclear staining (Hoechst 33342) is in blue ( $\times 63$  original magnification). *C*, Mincle is expressed at the leading edge of resting macrophages (*left panel*) and in the nascent phagocytic cup of primary BMM exposed to *C. albicans* yeasts (*right panel*).

domain of mouse Mincle was expressed as a recombinant histidine-tagged protein and used to test a direct interaction with yeast. To titrate the interaction between the receptor, Mincle, and its ligand, soluble yeast extract was prepared and equivalent quantities were used to coat an ELISA plate. Fig. 5A shows a titration curve of the Mincle protein against three different strains of *C. albicans*, as well as the yeast *S. cerevisiae*. Mincle bound equally to all yeasts at high concentrations of soluble yeast extract, but when concentrations were lowered, *Candida* isolate 3683 (an oral isolate) showed lower affinity for Mincle than *Candida* isolates 3630 and SC5314.

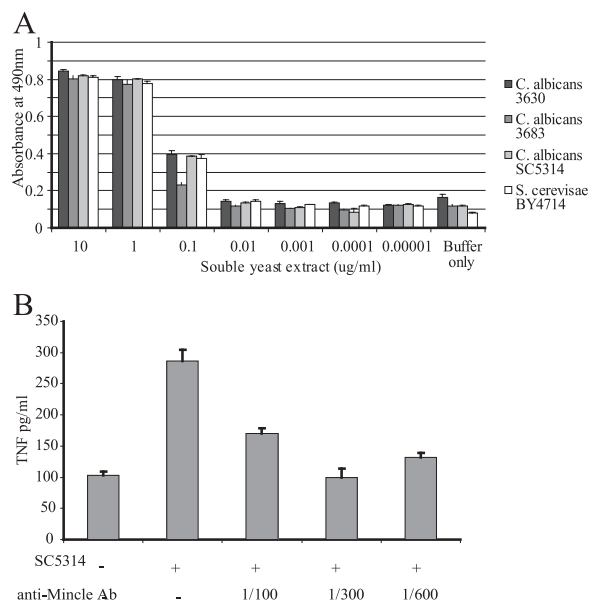
A direct interaction between Mincle and *Candida* was further characterized by using the Ab to block the C-type lectin domain of native Mincle in RAW264.7 cells before infection with *Candida*. Preincubation of RAW264.7 cells with the Mincle Ab

resulted in partial, statistically significant ( $p < 0.05$  by Student's *t* test) inhibition of TNF- $\alpha$  production in response to live *Candida* in a dose-dependent manner (Fig. 5B). The most prominent reduction occurred at a 1/300 dilution of original IgG Ab with up to 60% reduction in TNF- $\alpha$  secretion ( $p < 0.05$  by Student's *t* test).

#### Phenotype of Mincle gene KO mice

The ex vivo experiments conducted above were strongly indicative of a role for Mincle in the detection of *Candida albicans* by the innate immune system, but the analysis of Mincle's role in *Candida* susceptibility required the generation of Mincle KO mice. The recombinant KO mouse lines Clec4e<sup>MNA</sup> and Clec4e<sup>MNB</sup> carry a germline deletion of exons 3–4 of Clec4e on the C57BL/6J background, ablating Mincle protein expression. We confirmed by

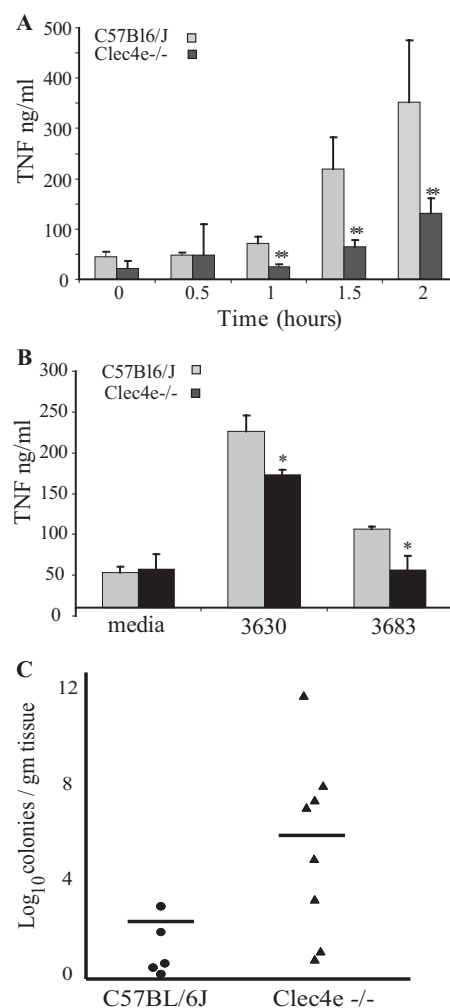




**FIGURE 5.** A, Binding of recombinant Mincle protein to soluble extracts of heat-killed yeasts. Three strains of *C. albicans* (3630: black bars; 3683: dark gray bars; and SC5314: light gray bars) and the *S. cerevisiae* strain BY4714 (white bars) bound to the C-type lectin domain of mouse Mincle. Data are presented as the mean  $\pm$  SE of two replicates. B, TNF- $\alpha$  production by RAW264.7 cells preincubated with Mincle Ab for 15 min before exposure to *Candida* isolate SC5314 for 30 min. Data shown are representative of five separate experiments, and are presented as the mean (picograms per milliliter)  $\pm$  SD of eight replicates. Lane 1, Negative control (medium only); lane 2, SC5314, no Ab; lane 3, preincubation with 1/100 Mincle Ab + SC5314; lane 4, preincubation with 1/300 Mincle Ab + SC5314; lane 5, preincubation 1/600 Mincle Ab + SC5314.

RT-PCR that BMM derived from both of the KO mouse lines carried the null allele (Fig. 1C), and confirmed lack of expression of the Mincle protein in KO macrophages by immunofluorescence (Fig. 3). White cell counts from Clec4e<sup>MNA</sup> and Clec4e<sup>MNB</sup> mice were generally low, but within normal ranges reported for C57BL/6J mice (from the Mouse Phenome Database <http://www.jax.org/phenome> (30, 31)). In this current study, circulating white blood cell counts of Clec4e<sup>MNA</sup> and Clec4e<sup>MNB</sup> mice were 20% lower than those of control C57BL/6J mice ( $p < 0.05$ ), and this depression was observed across neutrophil (25% lower), monocyte (15% lower), and lymphocyte (19% lower) counts. Nevertheless, the data were still within the normal range for C57BL mice, so the mice were not considered to be demonstrating an immune cell deficiency. Cells derived from the bone marrow of KO animals were normally responsive to rM-CSF ( $n = 16$ , across four independent experimental series), as no differences in macrophage number or morphology after 5 days of differentiation *ex vivo* were observed when compared with control C57BL/6J BMMs ( $n = 16$ ) grown concurrently with the KO cells. Neither cell-mediated immune function, as measured by delayed-type hypersensitivity responses, nor Ig production by the KO animals, differed significantly from that of controls.

The mice were grossly anatomically normal, although histological examination of the Clec4e<sup>MNA</sup> line showed evidence of abnormal heart valves in 12 of the 14 null animals. The heart was globular and the heart valves showed increased amounts of extracellular matrix. These accumulations resulted in globular endings of the valve leaflets, which could lead to poor closure of the valves in diastole. This preliminary histology data may indicate an endogenous ligand for Mincle, as well as a role in heart valve development.



**FIGURE 6.** MNB (Clec4e<sup>-/-</sup>) mice demonstrate reduced cytokine production after challenge with *C. albicans*, and are more susceptible to systemic candidiasis. A, Time course of TNF- $\alpha$  production in Clec4e<sup>-/-</sup> and C57BL/6J BMM in response to *C. albicans*. The data represent means  $\pm$  SE of eight independent determinations. BMM from Clec4e<sup>-/-</sup> mice (■) have significantly lower TNF production (\*\*, Student *t* test,  $p < 0.001$ ) compared with C57BL/6J mice (□). The data indicate a delay in response to *Candida* in the Clec4e<sup>-/-</sup> animals, with a measurable increase in TNF production by 1 h in the WT BMM, as compared with 1.5 h in the Clec4e<sup>-/-</sup> BMM. B, TNF production by BMM from Clec4e<sup>-/-</sup> mice (■) and control C57BL/6J mice (□) after stimulation with *C. albicans* isolate 3630 for 60 min, compared with medium alone. Data shown are representative of five separate experiments using pooled BMM from four cultures each of control and KO mouse. Results are presented as the mean (picograms per milliliter)  $\pm$  SD of eight replicates. \*, Twenty-five percent reduction in TNF production,  $p < 0.05$ , by Student's two-tailed *t* test. C, Increased colonization of the kidneys in MNB (Clec4e<sup>-/-</sup>) mice infected *i.v.* with  $3 \times 10^5$  *C. albicans* 3630. The magnitude of the fungal burden in the KO mice is significantly greater than in the controls ( $p < 0.05$  by Student's *t* test).

Original data files from the mouse phenotyping screens and images are available from the Functional Glycomics Consortium website (Nature Gateway, 2007 Mouse Line Phenotype Analysis; <http://www.functionalglycomics.org/glycomics/publicdata/phenotyping.jsp>).

#### Primary macrophages lacking Mincle have reduced responses to *C. albicans*

To determine the effect of Mincle deletion on inflammatory responses of KO macrophages, TNF- $\alpha$  was assayed from the supernatant of naive or *Candida*-stimulated BMMs generated from

Table II. Phagocytosis of *C. albicans* strain 3630 by RAW264.7 cells is independent of Mincle availability

Ab Dilution	% of Macrophages Phagocytosing Yeast	Phagocytic Index
0	24.34 ± 5.89	39.43 ± 14.03
1/100	23.67 ± 4.07	40.48 ± 7.45
1/300	26.63 ± 3.72	42.87 ± 7.33
1/3000	25.14 ± 4.18	41.23 ± 9.24
1/30000	18.59 ± 5.74	30.67 ± 11.83
Control <sup>a</sup>	25.25 ± 0.105	40.65 ± 2.81

<sup>a</sup> The working concentration of the Mincle Ab was 0.6 mg/ml<sup>-1</sup>, and the anti-IgG was used at 1/500. The data represent the mean ± SEM of three experiments.

Clec4e<sup>MNB</sup> KO lines, as well as isogenic C57BL/6J controls. Clec4e<sup>MNB</sup> BMM consistently produced at least 25–30% less TNF- $\alpha$  than isogenic controls in response to *Candida* stimulation (Fig. 6A); however, in some experiments, the yeast-stimulated KO BMM produced no TNF- $\alpha$  at all. Markedly less TNF- $\alpha$  was produced after stimulation with *C. albicans* isolate 3683 in comparison to 3630 (Fig. 6B). In separate experiments, we observed no significant difference in TNF- $\alpha$  production between Clec4e<sup>MNB</sup> and WT (C57BL/6) mice in response to 50 ng/ml LPS (average 900 pg/ml ± 2% from either mouse line) or 100  $\mu$ g/ml zymosan (average 850 pg/ml ± 4% from either mouse line).

#### Mincle is not a phagocytic receptor

As attenuated cytokine production in the absence of Mincle might have been a consequence of a defect in phagocytosis, the effect of Ab blocking on the uptake of yeast into the phagosome was examined. Blocking the Mincle C-type lectin domain by the Mincle Ab had no effect on the number of 3630 yeast particles phagocytosed by RAW264.7 cells (Table II), nor the number of macrophages clearing yeast. Similarly, *Candida* uptake by primary BMMs from the Clec4e<sup>MNB</sup> mouse lines was not significantly different to the isogenic C57BL/6J controls (Table III). These data demonstrated that the regulation of TNF- $\alpha$  production by Mincle was not dependent on yeast uptake in vitro or ex vivo cell systems, but rather indicated that Mincle may mediate inflammatory signaling pathways that converge on TNF- $\alpha$  production.

#### Mice lacking Mincle show increased susceptibility to systemic *C. albicans* infections

We finally examined the ability of Mincle KO mice to control *Candida* infection after a systemic challenge. No mice died within the 5-day period of observation, but the magnitude of the fungal burden in the kidneys of the KO mice was significantly greater ( $p < 0.05$ ) than in the controls (Fig. 6C). This observation provides compelling evidence for an essential role in

Table III. Phagocytosis of *C. albicans*, isolates 3630 and 3683, is not impaired in BMM isolated from MNB (Clec4e<sup>-/-</sup>) mice compared to C57BL/6J controls

Mouse Strain/ <i>Candida</i> Strain	% of Macrophages Phagocytosing Yeast	Phagocytic Index
Clec4e <sup>MNB</sup> /C. <i>albicans</i> 3630	51.6	175
Clec4e <sup>MNB</sup> /C. <i>albicans</i> 3683	66.9	316
C57BL/6J/C. <i>albicans</i> 3630	58.7	203
C57BL/6J/C. <i>albicans</i> 3683	48.4	221

innate immune recognition and clearance of the yeast infection in vivo.

## Discussion

The initial immune response to a *Candida* infection is dictated by the set of *Candida*-inducible genes activated by the initial interaction with specific PRRs on the surface of the responding cell. The present study has identified the C-type lectin Clec4e (Mincle) and *Tlr2* as genes of interest, and we have now shown that Mincle recognizes clinical isolates of *C. albicans*, contributes substantially to the production of the inflammatory cytokine TNF- $\alpha$  in the host response to *Candida*, and plays a significant part in recovery from infection.

The ligand for Mincle has not yet been identified, but our preliminary data suggest that it does not interact with either yeast zymosans or  $\beta$ -glucans; however, recombinant Mincle protein did bind to the soluble component of both heat-killed yeasts *Candida* and *Saccharomyces*. Glycosylation in *C. albicans* and *S. cerevisiae* involves mostly oligosaccharides predominantly composed of mannose residues (32), but they can also contain small amounts of the sugars *N*-acetylglucosamine, *N*-acetylgalactosamine, as well as sialic acid and rhamnose, among others (33, 34). Mincle contains the amino acids EPN, a common carbohydrate-binding motif, which is also found in Dectin-2 and CD23 (17, 35). This motif indicates Mincle may have a binding preference for mannose derivatives or *N*-acetylglucosamine residues. The observation that Mincle bound the two strains of *C. albicans* 3683 and 3630 with different affinity is consistent with a carbohydrate ligand that is differentially expressed by these yeasts. This may represent the basis for the discrimination of different yeasts by Mincle.

Exposure of macrophages to *C. albicans* in vitro results in engagement of Mincle with its ligand, and rapid recruitment to the phagosome. Interestingly, hyphal penetration into the cell (Fig. 4B) did not appear to stimulate local accumulation of Mincle, indicating that the stimulus for translocation was specific triggering of the receptor. Abrogation of Mincle-pathogen interactions by Ab blocking of the binding domain did not alter uptake of *Candida* by macrophages, nor was it altered in BMM from Mincle KO mouse lines. Although inflammatory cytokine production in macrophages is tightly coupled to phagocytosis, signaling events mediated by Mincle were dependent on specificity of recognition, as in the absence of Mincle, or upon blocking of available protein by a neutralizing Ab, *Candida*-induced TNF- $\alpha$  production was reduced, whereas TNF- $\alpha$  production by the same cells in response to LPS was unaltered. The appearance of Mincle in the phagosome, and the accompanying reduction in secretion of the inflammatory cytokine TNF- $\alpha$  in response to *C. albicans* in the presence of neutralizing Ab, indicates that Mincle is unlikely to be an endocytic PRR. Instead, we predict that Mincle is more likely to function as an accessory PRR, that may be involved in the initial detection and signaling response to *Candida* from within the phagosome.

Mincle is a transcriptional target of NF-IL6 (C/EBP $\beta$ ) and is one of only two genes whose induction is defective in the absence of this transcription factor (16, 36). Like Mincle KO mice, mice lacking C/EBP $\beta$  showed increased susceptibility to *C. albicans* infection (37), and macrophages from these animals displayed inverse regulation of IL-12 p40 and IL-12 p35 (38). In this context, it may not be coincidental that IL-12 has been shown to be essential for recovery from oral candidiasis in mice (39). Recovery from systemic candidiasis is dependent neither on IL-12 (29) nor on the presence of functional T lymphocytes (39), consistent with our observations that Mincle KO mice

demonstrated increased *Candida* colonization of the kidneys. This is suggestive of impaired clearance by the innate immune system. This could be attributable to a defect in recognition and killing by myeloid cells, but may also reflect poor recruitment of effector cells through reduced cytokine production. Although neutrophils are essential for host resistance against systemic candidiasis (40), the small decrease in leukocyte numbers in Mincle KO mice is unlikely to account for their increased susceptibility to infection. Further investigations into the expression of Mincle on granulocytes, and its function in these cells, are currently being undertaken.

Mincle has been reported to be expressed predominantly on cells of the myeloid lineage, such as macrophages, dendritic cells, and B cells (41), and also on microglia in the brain (42). Although this pattern of expression is broadly similar to both Dectin-1 (43) and Dectin-2 (44), we have demonstrated that mouse BMM constitutively express Mincle but not Dectin-1 or -2, and that its expression is increased after stimulation with *C. albicans*. It is also induced by IFN (18), and up-regulated by both LPS signaling (16), and Semliki Forest Virus infection (42). The presence of intact cellular and humoral memory responses in Mincle KO mice suggests that it may play a lesser role (if any) in Ag presentation and the initiation of adaptive immune responses.

In conclusion, Mincle forms part of an emerging class of PRR—the C-type lectins—that are increasingly demonstrating an important role for glycobiology in host responses to infection. Mincle is a receptor for *C. albicans*, and is rapidly induced in macrophages exposed to the yeast. Inflammatory cytokines generated by the interaction of Mincle with its ligand may act in a positive feedback loop to increase its expression, and its potential to discriminate carbohydrate moieties on the yeast cell wall may enhance the selectivity and efficiency of the innate immune response. Carbohydrate recognition may thus act as part of a generalized “call to arms” in response to any infection, allowing the macrophage to detect multiple pathogens likely to be present when a mucosal or epidermal barrier is breached.

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## Disclosures

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